The Journal of Nutrition. First published ahead of print October 16, 2013 as doi: 10.3945/jn.113.183004.

The Journal of Nutrition
Nutrient Physiology, Metabolism, and Nutrient-Nutrient Interactions

Colonic Hydrogen Generated from Fructan Diffuses into the Abdominal Cavity and Reduces Adipose mRNA Abundance of Cytokines in Rats[1–3]

Naomichi Nishimura,[4]* Hiroki Tanabe,[4] Misato Adachi,[4] Tatsuro Yamamoto,[4] and Michihiro Fukushima[5]

[4]Department of Nutritional Sciences, Faculty of Health and Welfare Science, Nayoro City University, Nayoro, Hokkaido, Japan; and
[5]Department of Animal Science, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan

Abstract

Hydrogen (H_2) protects against inflammation-induced oxidative stress. Nondigestible saccharides (NDSs) enhance colonic H_2 production. We examined whether colonic H_2 transfers to tissues in the abdominal cavity and whether it affects expression of proinflammatory cytokines. In Expts. 1 and 2, rats were fed diets containing fructooligosaccharides [FOSs; 25 (Expt. 1) and 50 g/kg (Expts. 1 and 2)] for 7 and 14 d, respectively. The no-FOS diet was used as the control diet. At the end of the experiment, H_2 excretion and the portal H_2 concentration were significantly greater in the FOS group than in the control group. In the FOS group, the arterial H_2 concentration was no more than 1.5% of the portal H_2 concentration (P = 0.03). The H_2 concentration in abdominal cavity tissues, especially adipose tissue, in the FOS group was 5.6- to 43-fold of that in the control group ($P < 0.05$). The H_2 content in the abdominal cavity in the FOS group was 11-fold of that in the control group ($P < 0.05$). In Expt. 3, rats were fed a high-fat diet containing FOS and inulin (50 g/kg) for 28 d. The area under the curve for H_2 excretion between 0 and 28 d and portal and adipose H_2 concentrations were significantly higher in the FOS and inulin groups than in the high-fat control group. Adipose mRNA abundance of nuclear factor kappa-light-chain-enhancer of activated B cells 1 was lower in the FOS group than in the control group (P = 0.02) and those of interleukin-6 and chemokine (C-C motif) ligand 2 tended to be lower ($P < 0.11$). Colonic H_2 generated from NDS diffuses to the abdominal cavity before transferring to abdominal tissues. Reduced cytokine expression by FOS feeding might be dependent on increased colonic H_2. Colonic H_2 may have important implications in the suppressive effect on metabolic syndrome via oxidative stress. J. Nutr. doi: 10.3945/jn.113.183004.

Introduction

Excessive oxidative stress can trigger the onset and progression of various metabolic syndromes such as diabetes mellitus, hypertension, obesity, dyslipidemia, and proinflammatory status (1,2). Research to identify and elucidate the function of antioxidant substances to reduce oxidative stress is under way (3). The effect of hydrogen (H_2) in reducing oxidative stress has been clarified. The reduction of oxidative stress in vivo has been confirmed in rodents administered H_2 through inhalation of H_2 gas and drinking H_2 water (4–9), suggesting that H_2 may constitute a powerful, safe antioxidant in vivo. The consumption of nondigestible saccharides (NDSs)[6] such as oligosaccharide, dietary fiber, and resistant starch that form fermentation substrates in the large intestine results in the generation of large quantities of H_2 (10–12). Our previous studies in a hepatic ischemia-reperfusion rat model clarified that H_2 generated by fermentation in the large intestine possesses the same antioxidative action as H_2 obtained through drinking water or inhalation (13).

A large proportion of H_2 generated in the large intestine is excreted in the breath and flatus. However, the shift of generated H_2 to various organs and tissues remains unclear. Low concentrations of H_2 can be detected in the arterial blood, because almost all of the H_2 absorbed into the portal blood is excreted by lung gas exchange. H_2 is the smallest molecule known to exist

[1] Supported in part by a Regional Innovation Strategy Support Program ''Tokachi Agricultural Bioscience Cluster'' of the Ministry of Education, Culture, Sports, Science and Technology, Japan.
[2] Author disclosures: N. Nishimura, H. Tanabe, M. Adachi, T. Yamamoto, and M. Fukushima, no conflicts of interest.
[3] Supplemental Tables 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
* To whom correspondence should be addressed. E-mail: nishimura@nayoro.ac.jp.

[6] Abbreviations used: Ccl2, chemokine (C-C motif) ligand 2; DP, degree of polymerization; FOS, fructooligosaccharide; HFC, high-fat, control group; HFF, group fed the high-fat control diet supplemented with fructooligosaccharide; HFI, group fed the high-fat control diet supplemented with inulin; Il6, interleukin-6; NDS, nondigestible saccharide; NFC, normal-fat, control group; Nfκb, nuclear factor kappa-light-chain-enhancer of activated B cells; Rps18, ribosomal protein S18; Tnfα, tumor necrosis factor-α.

Manuscript received July 25, 2013. Initial review completed August 1, 2013. Revision accepted September 30, 2013.
doi: 10.3945/jn.113.183004.

(covalent radius and Van der Waals radius are 31 and 120 pm, respectively). H_2 is a nonpolar molecule and its solubility in fat is 3–5 times greater than that in water (14,15). From these findings, H_2 should be able to pass through the wall of the large intestine and portal vein and then diffuse into the abdominal cavity. We propose that some of the H_2 generated in the large intestine transfers into the abdominal cavity. High H_2 generation in the large intestine could be an effective means to suppress oxidative stress in vivo.

Obesity is associated with low-grade inflammation in adipose tissue (1). The inflammation can be attributed to increased oxidative stress and secretion of cytokines and chemokines, resulting in insulin resistance (1). High H_2 concentrations in the adipose tissues could be a potential factor in preventing inflammation. The fermentation of fructans, such as FOS and inulin, in the large intestine generates a higher amount of H_2 than lactulose, galactosyl sucrose, and isomalto-oligosaccharide fermentation (16,17). Recently, we found that fructan and galactan, but not glucan, were good fermentation substrates by which abundant H_2 was generated (N. Nishimura, H. Tanabe, A. Shishido, T. Yamamoto, M. Fukushima, unpublished data). Therefore, in the present study, we examined whether H_2 generated by fermentation of fructooligosaccharide (FOS) and inulin in the large intestine transfers to the tissues in the abdominal cavity and whether H_2 affects the expression of proinflammatory cytokines in adipose tissues.

Materials and Methods

Samples. FOS [Meioligo-P; degree of polymerization (DP), 2–4] and inulin (Tokachi-inulin derived from chicory; DP, 2–54; mean DP, 14) were kindly supplied by Meiji Food Materia and Nippon Beet Sugar Manufacturing, respectively.

Rats and diets. The study was approved by Nayoro City University Animal Care and Use Committee and the rats were maintained in accordance with guidelines for the care and use of laboratory animals, Nayoro City University. Male Sprague-Dawley rats weighing 200–230 g (7 wk old; Expts. 1 and 2) and 260–280 g (8 wk old; Expt. 3) were obtained from Japan SLC (Haruno colony). They were housed in individual stainless steel cages with screen bottoms in a room maintained at 23 ± 1°C with humidity ranging from 50 to 70% and under lighting conditions of 12 h light/12 h dark (0700–1900 h) daily. Coprophagy was allowed. The rats were acclimatized by feeding a 25% casein control diet for 3 d in Expts. 1 and 2 and an normal-fat control diet (NFC) diet (12% fat:energy ratio) for 10 d in Expt. 3 (**Supplemental Table 1**). At the end of the experimental period, rats were killed by cervical dislocation under anesthesia after sampling of blood and tissues.

Expt. 1. To determine the movement of H_2 generated in the large intestine, we examined the changes in H_2 concentration that occurred in various tissues in rats fed a diet containing different amounts of FOS. After the acclimation period, breath and flatus H_2 excretion per 5 min was measured by placing the rats inside a sealed polypropylene chamber for 5 min. GC (Biogas analyzer BAS-1000; Mitleben) was used to determine breath and flatus H_2 excretion. Eighteen rats were assigned to 3 groups (n = 6) based on breath and flatus H_2 excretion and body weight. The first group was administered the control diet and the second and third groups were administered diets containing FOS (25 or 50 g/kg) for 7 d. FOS supplementation was achieved by replacement of an equal weight of cornstarch in the control diet.

Expt. 2. To determine the route of H_2 generated in the large intestine to the abdominal tissues, including the adipose tissues, we examined the changes in H_2 concentration in the abdominal cavity and aortic blood in rats fed a diet containing FOS. After the acclimation period, breath and flatus H_2 excretion per 5 min was measured using the same method as in Expt. 1. Twelve rats were assigned to 2 groups (n = 6) based on breath and flatus H_2 excretion and body weight. The first group was administered the control diet and the second group was administered the diet containing FOS (50 g/kg) for 14 d. FOS supplementation was achieved by replacement of an equal weight of cornstarch in the control diet.

Expt. 3. To determine the effect of H_2 generated in the large intestine on obesity-induced, low-grade, chronic inflammation in adipose tissue, we examined the change in mRNA abundance of inflammatory cytokines and chemokines in perirenal adipose tissue in rats fed a high-fat control diet (HFC; 46% fat:energy ratio) containing FOS and inulin (50 g/kg) (Supplemental Table 1). FOS and inulin supplementation was achieved by replacement of an equal weight of cornstarch in the HFC diet. After the acclimation period, breath and flatus H_2 excretion per 5 min was measured using the same method as above. Thirty-two rats were assigned to 4 groups (n = 8) based on breath and flatus H_2 excretion and body weight. The first, second, third and fourth groups were administered the NFC diet, the HFC diet, and the HFC diet containing FOS or inulin for 28 d, respectively. A power analysis of H_2 excretion on d 28 was conducted using SAS JMP (version 9.0.2). The least significant number and statistical power were 17.3 and 0.479, respectively.

Sampling. On the last day of the experiment, rats were anesthetized with sodium pentobarbital (50 mg/kg body weight). In Expt. 1, after immediate laparotomy, 1 mL of blood was removed from the portal vein and the abdominal aorta into sealed heparin vials for H_2 analysis. Also, ~1 g of liver, spleen, kidney, perirenal adipose tissue, testis, epididymal adipose tissue, lung, and brain were immediately removed into sealed vials for H_2 analysis. An appropriate volume sample of the gaseous phase was withdrawn using a gas-tight syringe and H_2 concentration was determined using GC (Biogas analyzer BAS-1000; Mitleben). In Expt. 2, rats were i.p. injected with 3 mL of sterilized saline. Following 30 s of standing, we immediately performed laparotomy and then 1 mL of injected saline was collected into sealed vials. Then 1 mL of blood was removed from the portal vein and the abdominal aorta into sealed heparin vials for H_2 analysis. Also, 1 g of liver, perirenal adipose tissue, and epididymal adipose tissue were removed into sealed vials for H_2 analysis. The H_2 concentration was determined as above. In Expt. 3, after immediate laparotomy, 1 mL of blood was removed from the portal vein into sealed heparin vials and then ~1 g of perirenal adipose tissue was removed into sealed vials for H_2 analysis. H_2 concentration was determined as above. An aliquot of the remaining tissue was stored at −30°C for RNA extraction after treatment with RNAlater (Life Technologies). The cecum was removed and weighed and the cecal content was collected and stored at –80°C in air-tight tubes until SCFA and bacterial DNA analysis. The cecal wall was blotted and weighed after rinsing in an ice-cold saline solution.

Real-time RT-PCR analysis of mRNA abundance of Nfκb1, Il6, Tnfα, and Ccl2. Total RNA was extracted from the perirenal adipose tissue using the RNeasy Mini kit (Qiagen) and reverse-transcribed using the Reverse Transcriptase kit (PrimeScript RT reagent kit, Takara Bio). Genomic DNA was removed by on-column DNase digestion using the RNase-Free DNase Set (Qiagen). The primers for rat Il6, chemokine (C-C motif) ligand 2 (Ccl2), tumor necrosis factor (Tnfα), and ribosomal protein S18 (Rps18) were designed by TaKaRa Perfect Real Time Support System (Takara); the sequences are listed in **Supplemental Table 2**. Rps18 was used as an endogenous control. Primers for the nuclear factor kappa-light-chain-enhancer of activated B cells (Nfκb1) gene (accession no. NC_005101.3) (18) were designed to cross an exon-intron junction using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi). Real-time PCR was carried out in duplicate for each sample on an Agilent Mx3000P (Agilent Technologies) using the SYBR Premix Ex Taq II kit (Takara). The specificity of the PCRs was determined by dissociation curve analysis. For each sample, results were normalized to Rps18 values and calculated as arbitrary units.

qPCR of microbial cecal content. Bacterial DNA was isolated from frozen cecal contents using a DNA extraction kit (ISOFECAL for Beads Beating kit, Nippon Gene) according to the manufacturer's protocol,

except for sample disruption. Frozen cecal contents (200 mg) were disrupted at 2700 rpm for 90 s using a Multi-Beads shocker (MB601U, Yasui Kikai). Extracted DNA samples were dissolved in DNase-free Tris-EDTA buffer (pH 8.0; 10 mmol/L Tris-HCl, 1 mmol/L EDTA) and stored at –30°C until qPCR analyses. The PCR was performed in a total volume of 20 μL. All bacteria were detected using the SYBR Premix Ex Taq II (Takara), with 200 nmol/L each of respective primers and <1 ng DNA for each reaction. As shown in Supplemental Table 2, the primers used to detect the bacteria were based on 16S rRNA gene sequences (19). The PCR amplification reactions were carried out according to the method of Guo et al. (19). Detection was carried out on an Agilent Mx3000P (Agilent Technologies). Ten-fold serial dilutions of known quantities of TA cloning vector (pCR2.1-TOPO, Life Technologies) with inserted 16S rDNA from *Lactobacillus acidophilus* (JCM1132) or *Bacteroides vulgatus* (JCM5826) were used to generate standard curves to count copies of total bacteria of *Firmicutes* or *Bacteroidetes*, respectively. Absolute rDNA abundance was calculated based on the standard curves. Each assay was performed in duplicate in the same analysis.

SCFA analysis. After homogenization of cecal contents, cecal organic acids (acetate, propionate, and n-butyrate) were measured using an HPLC system (LC-10A, Shimadzu) equipped with a Shim-pack SCR-102H column (8 mm i.d. × 30 cm long, Shimadzu) and an electroconductivity detector (CDD-6A, Shimadzu) (20). Briefly, ~300 mg cecal content was homogenized in 2 mL of 10 mmol/L NaOH containing 0.5 g/L crotonic acid as an internal standard and then centrifuged at 10,000 × *g* at 4°C for 15 min. The supernatant was subjected to HPLC analysis after deproteinization with an equal volume of chloroform.

Statistical analysis. Values obtained from the experiments were expressed as means ± SEMs. Data were subjected to Bartlett's test for homogeneity of variances, and unequal variances were stabilized by log transformation. For samples with equal variances, 1-factor ANOVA was used, followed by Tukey-Kramer post hoc test for multiple comparisons between individual group means. If sample variances were still unequal after log transformation, we used the Steel-Dwass test. Furthermore, we used the Student's *t* test for data in Expt. 2 and between the NFC and HFC groups in Expt. 3. Repeated-measures, 2-factor ANOVA was used to analyze the change in H_2 excretion among the 3 high-fat groups across time. H_2 excretion within an individual group was compared with repeated-measures, 1-factor ANOVA. Comparisons among the 3 high-fat groups at specific time points were made by Tukey-Kramer test. Pearson correlation tests were used to evaluate the association between portal H_2 concentration and adipose mRNA abundance of cytokines in the 3 high-fat groups. All statistical analyses were performed using SAS JMP software (version 9.0.2). Significance was defined as $P < 0.05$.

Results

Expt. 1. Body weight gain and food intake did not differ among the 3 groups (data not shown). Coprophagy was not observed. Breath and flatus H_2 excretion and portal H_2 concentration were dose dependently higher in rats receiving dietary FOS supplementation (**Fig. 1***A*); however, no significant difference was observed in the aortic H_2 concentration. The H_2 concentration in the liver, kidney, and adipose tissues dose dependently increased by FOS feeding compared with the control group and in the 5% FOS group was 23-, 43-, and 37-fold of the respective value in the control group ($P = 0.0141$).

Expt. 2. Body weight gain and food intake did not differ among the groups (data not shown). Coprophagy was not observed. As in Expt. 1, breath and flatus H_2 excretion and portal H_2 concentration were significantly higher in the FOS group compared with the control group (Fig. 1*B*). Only small aortic H_2 concentrations were detected. In addition, high H_2 concentrations in the liver and perirenal fat were detected in rats fed the FOS diet compared with the control group. Interestingly, H_2 concentrations in the abdominal cavity were ~10 times greater in the FOS group than in the control group (Fig. 1*B*).

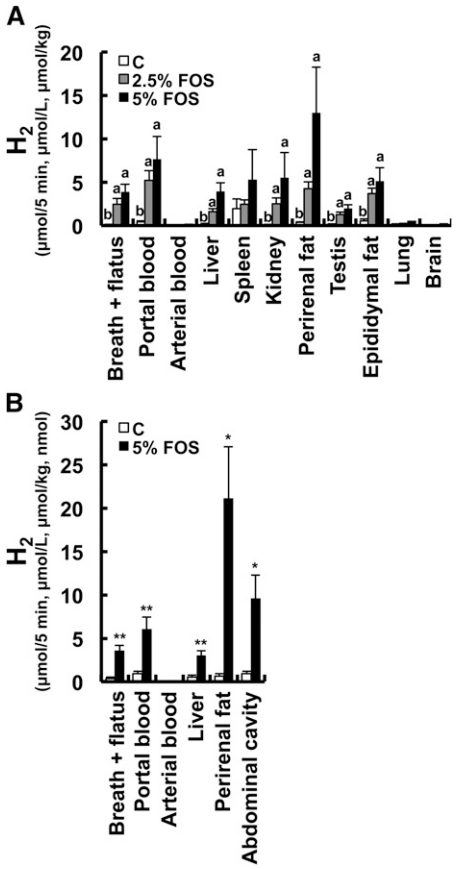

FIGURE 1 Breath and flatus H_2 excretion and portal blood, aorta, abdominal cavity, and tissue H_2 concentrations in rats fed diets containing 0–5% FOS in Expts. 1 (*A*) and 2 (*B*). Values are means ± SEMs, $n = 6$. In *A*, labeled means for a variable without a common letter differ, $P < 0.05$. In *B*, asterisks indicate different from the control group: $^*P < 0.05$, $^{**}P < 0.01$. Units for H_2 in breath and flatus are μmol/5 min; in blood, μmol/L; in tissue, μmol/kg, and in the abdominal cavity, nmol. C, rats fed the control diet; FOS, fructooligosaccharide; 2.5% FOS and 5% FOS, rats fed the control diet containing 2.5% and 5% fructooligosaccharides, respectively.

Expt. 3. Food intake in the HFC group was significantly lower than that in the NFC group, but energy intake and body weight gain did not differ between the 2 groups (**Table 1**). There were no significant differences in food intake of the 3 high-fat groups. Body weight gain tended to be lower in the group fed the high-fat control diet supplemented with inulin (HFI) than in the HFC group ($P = 0.09$), but there was no significant difference among the 3 high-fat groups. Coprophagy was not observed. Breath and flatus H_2 excretion was significantly higher in the group fed the high-fat control diet supplemented with fructooligosaccharide (HFF) and HFI groups than in the HFC group, and the high level was maintained for 28 d (**Fig. 2**). The AUC for 0–28 d in breath and flatus H_2 excretion ($AUC_{0–28d}$) and portal H_2 concentration were significantly greater in the HFF and HFI groups than in the HFC group (Table 1). Perirenal fat H_2 concentration was also higher in the HFF and HFI groups compared with the HFC group. Adipose mRNA abundance of Nfκb1 was significantly lower in the FOS group than in the control group ($P = 0.023$) and those of Il6 and Ccl2 tended to be lower ($P < 0.11$) (Table 1). Adipose mRNA abundance of Nfκb1 or Il6 was inversely correlated with portal H_2 concentration among the 24 rats fed

TABLE 1 Food and energy intake, body weight gain, breath and flatus H_2 excretion, portal and abdominal H_2 concentrations, and abdominal mRNA abundance in rats fed high-fat diets containing 5% fructan in Expt. 3[1]

	NFC	HFC	HFF	HFI
Food intake, *g/28 d*	654 ± 15	555 ± 22	526 ± 21	493 ± 21
Energy intake, *MJ/28 d*	10.2 ± 0.2	10.9 ± 0.4[a]	9.92 ± 0.4[ab]	9.3 ± 0.4[b]
Body weight gain, *g/28 d*	133 ± 5	156 ± 10	137 ± 8	125 ± 11
Breath and flatus H_2 excretion $AUC_{0-28\ d}$, *mmol*	1.00 ± 0.45	1.10 ± 0.27[b]	96.7 ± 17.4[a]	55.5 ± 10.2[a]
Portal H_2, *μmol/L*	0.542 ± 0.286	0.656 ± 0.231[b]	8.82 ± 1.06[a]	7.64 ± 1.01[a]
Perirenal fat				
Weight, *g/100 g body weight*	2.38 ± 0.15	2.85 ± 0.17	2.91 ± 0.14	2.40 ± 0.15
H_2, *μmol/kg*	0.654 ± 0.156	0.961 ± 0.223[b]	9.04 ± 2.95[a]	12.7 ± 3.5[a]
mRNA abundance, *fold of that in NFC*				
Il6	1.00 ± 0.40	0.987 ± 0.494	0.117 ± 0.022	0.194 ± 0.047
Tnfα	1.00 ± 0.11	0.723 ± 0.121	0.497 ± 0.050	0.717 ± 0.094
Ccl2	1.00 ± 0.15	1.03 ± 0.20	0.580 ± 0.042	0.777 ± 0.140
Nfκb	1.00 ± 0.04	1.09 ± 0.05[a]	0.883 ± 0.059[b]	0.951 ± 0.040[ab]

[1] Values are means ± SEMs, $n = 8$. No significant difference in the variables was observed between the NFC and HFC groups. Labeled means of 3 high-fat groups in a row without a common letter differ, $P < 0.05$. Ccl2, chemokine (C-C motif) ligand 2; HFC, high-fat, control group; HFF, group fed the high-fat control diet supplemented with 5% fructooligosaccharide; HFI, group fed the high-fat control diet supplemented with 5% inulin; Il6, interleukin-6; NFC, NFC, normal-fat, control group; Nfκb1, nuclear factor kappa-light-chain-enhancer of activated B cells; Tnfα, tumor necrosis factor-α.

the HFC, HFF, and HFI diets ($r = -0.42$, $P = 0.04$; $r = -0.40$, $P = 0.05$, respectively) and those of Ccl2 tended to be inversely correlated ($r = -0.37$; $P = 0.08$).

The weights of cecal tissue and contents were significantly greater in the HFF and HFI groups than in the HFC group (**Table 2**). The concentrations of acetate and butyrate in the cecal contents were higher in the HFF and HFI groups compared with the HFC group, but the propionate concentration did not differ among the 3 groups. The total number of bacteria was significantly higher in the HFI groups. The abundance of *Firmicutes* was significantly greater in the HFF and HFI groups than in the HFC group. In addition, the abundance of *Bacteroidetes* was significantly higher in the HFI group compared with the HFC and HFF groups.

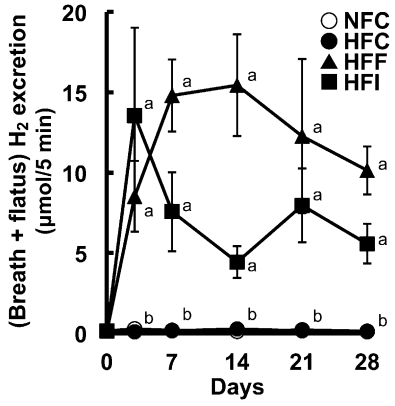

FIGURE 2 Change in breath and flatus H_2 excretion in rats fed the NFC, HFC, HFF, and HFI diets for 28 d in Expt. 3. Values are means, with their SEs represented by vertical bars, $n = 8$. Labeled means at a specific time point without a common letter differ, $P < 0.05$. No significant difference between the NFC and HFC was observed at a specific time point. Repeated-measures, 2-factor ANOVA was used to analyze H_2 excretion among the 3 high-fat groups across time (fructan, $P < 0.0001$; time, $P = 0.0001$; interaction, $P = 0.0008$). Within each group, all time points differed from d 0 ($P < 0.05$) but did not differ from one another. HFC, high-fat, control diet; HFF, high-fat control diet supplemented with fructooligosaccharide; HFI, high-fat control diet supplemented with inulin; NFC, normal-fat, control diet.

Discussion

Recently, inhaled H_2 gas and administered H_2 water have been reported to have an antioxidative effect in rodents with oxidative stress (4–10,12,14,19,21–25). In our previous study, we found that H_2 generated by fermentation of pectin and resistant starch in the large intestine alleviated hepatic oxidative stress induced by hepatic ischemia-reperfusion in rats (13,26). H_2 generated during intestinal fermentation has the advantage of delivering a continuous supply in vivo for as long as fermentation is sustained. However, the distribution in the body of H_2 generated in the large intestine has remained unclear, although much of the H_2 is excreted via breath and flatus (10–13).

In the present study, we found that part of the H_2 generated in the large intestine diffused into the abdominal cavity and was then localized in various tissues, especially the adipose tissues. The H_2 concentration in aortic blood was small, suggesting that H_2 in tissues of the abdominal cavity is predominantly dependent on distribution by diffusion from the large intestine. H_2 should be able to pass through the wall of the large intestine, because it is a very small molecule: the covalent radius and Van der Waals radius are 31 and 120 pm, respectively. Our results suggest that H_2 generated in the large intestine could have a potential effect on the redox levels in the abdominal tissues. H_2 is a nonpolar molecule and its solubility in fat is 3–5 times greater than that in water (14,15). This difference in solubility suggests why much of the H_2 accumulated in the adipose tissue compared with other tissues. To the best of our knowledge, there have been few studies demonstrating the diffusion of H_2 into the abdominal cavity from the hindgut and the localization of H_2 to adipose tissues. An increased concentration of H_2, an antioxidant molecule, in the adipose tissue by the administration of NDS, such as FOS, dietary fiber, and resistant starch, could be important, because the changes occurring in obese adipose tissue, including increased oxidative stress, leads to insulin resistance, and metabolic syndrome.

TABLE 2 Weight of cecal tissue and content, cecal SCFA concentration and bacterial counts and ratio in rats fed high-fat diets containing 5% fructan in Expt. 3[1]

	NFC	HFC	HFF	HFI
Tissue, *g*	0.796 ± 0.022	0.780 ± 0.017	1.11 ± 0.05	1.15 ± 0.05
Contents, *g*	2.85 ± 0.20	2.52 ± 0.14	4.94 ± 0.36	5.07 ± 0.34
SCFA, *μmol/g*				
Acetate	38.6 ± 1.7	36.0 ± 1.1^{b}	50.7 ± 2.7^{a}	50.6 ± 2.6^{a}
Propionate	11.6 ± 0.6**	8.99 ± 0.39	6.30 ± 1.44	8.91 ± 1.18
n-Butyrate	2.99 ± 0.36	2.35 ± 0.17^{b}	17.1 ± 1.1^{a}	14.5 ± 1.4^{a}
Bacterial counts, *log$_{10}$copies/g*				
Total bacteria	12.3 ± 0.1*	12.5 ± 0.0^{b}	12.6 ± 0.0^{ab}	12.7 ± 0.0^{a}
Firmicutes	11.8 ± 0.1*	12.0 ± 0.0^{b}	12.4 ± 0.1^{a}	12.3 ± 0.1^{a}
Bacteroidetes	12.1 ± 0.1	12.3 ± 0.1^{a}	11.8 ± 0.1^{b}	12.4 ± 0.0^{a}
Bacterial ratios				
Firmicutes:total bacteria	316 ± 21	301 ± 18^{b}	675 ± 26^{a}	372 ± 44^{b}
Bacteroidetes:total bacteria	577 ± 24	550 ± 14^{a}	185 ± 27^{b}	560 ± 43^{a}
Firmicutes:Bacteroidetes	565 ± 65	552 ± 44^{b}	4410 ± 840^{a}	721 ± 117^{b}

[1] Values are means ± SEMs, $n = 8$. Asterisks indicate different from the HFC group; * $P < 0.05$, ** $P < 0.01$. Labeled means of 3 high fat groups in a row without a common letter differ, $P < 0.05$. HFC, high-fat, control group; HFF, group fed the high-fat control diet supplemented with 5% fructooligosaccharide; HFI, group fed the high-fat control diet supplemented with 5% inulin; NFC, normal-fat, control group.

Obesity is recognized as a chronic low-grade inflammatory condition, characterized by abnormal adipocytokine production, such TNFα, IL6, and CCL2, and the activation of some proinflammatory signaling pathways, resulting in the induction of insulin resistance. Adipose inflammation is also associated with oxidative stress (1,27). The suppression of oxidative stress would be an important strategy in the protection against insulin resistance, because elevated oxidative stress promotes the secretion of proinflammatory adipocytokine. The localization of H_2, generated in the large intestine, in the adipose tissue could contribute to the alleviation of oxidative stress and inhibition of chronic inflammation. We suggest the preventive effect of dietary fiber and resistant starch on the development of metabolic syndrome is partially dependent on the alleviation of oxidative stress by H_2. A significant reduction and a trend of reduction in the mRNA abundance of Nfκb1 in perirenal adipose tissue were observed in rats fed the HFF and HFI diets, respectively, when compared with the HFC group. In addition, adipose mRNA abundance of Il6 in the HFF and HFI groups was 12% and 20% of that in the HFC group ($P = 0.11$ and 0.15), respectively. Our results suggest that increased H_2 concentration in adipose tissue by FOS and inulin feeding might suppress oxidative stress, thus alleviating inflammation.

Some investigators reported that feeding of NDS reduced adipose and leukocyte gene expression of proinflammatory cytokines and chemokines in rats and mice fed a high-fat diet (28, 29). Cani et al. (28) demonstrated that changes in gut microbiota controlled metabolic endotoxemia and inflammation by a mechanism that could increase intestinal permeability. In addition, they showed that an increased number of bifidobacteria, due to oligofructose feeding, contributed to the pathophysiological regulation of endotoxemia and the reduction in mRNA abundance of cytokines in visceral adipose tissue in high-fat–fed rats (28). Thus, these results suggest that it may be useful to develop specific strategies for modifying gut microbiota in favor of bifidobacteria to prevent the deleterious effects of high-fat diet-induced metabolic diseases. In our present study, the increased *Firmicutes*:*Bacteroidetes* ratio observed in rats fed FOS may contribute to reduced cytokine and chemokine expression. Most (90%) of the phylotypes of gut bacteria are members of 2 phyla: the *Bacteroidetes* (Gram-negative) and the *Firmicutes* (Gram-positive), so the change in this ratio represents the modification of cecal microbiota after FOS treatment. However, further investigation is required to verify that the observed increase in the *Firmicutes*:*Bacteroidetes* ratio after FOS feeding could be mediated by the types of bacteria.

SCFAs, fermentation products of NDS by gut bacteria, have been reported to be modulators of cytokine and chemokine expression (29–31). In the present study, FOS and inulin enhanced the production of SCFA in rats fed high-fat diets. However, after feeding dietary fiber the increase in these acids in arterial blood could be barely detected (32). Unlike the H_2 molecule, SCFAs scarcely diffuse to the abdominal cavity from the lumen of the large intestine, suggesting that most of the SCFA would not be supplied to the adipose tissues. In a recent study, small amounts of acetate, propionate, and butyrate in human arterial blood of ~200, 3, and 3 μmol/L, respectively, were detected using a liquid chromatography-MS technique (33). In the present study, arterial SCFAs were not determined. Therefore, the possibility that SCFAs mediate adipose mRNA expression of cytokines cannot be denied. The impact of SCFA on adipose cytokine expression remains unclear and further study is required to determine the effect of various fermentation products derived from NDS, including SCFAs.

Fructans were added to the diet at doses of 2.5% and 5% in this study. This corresponds to 10–20 g of fructans if dry matter in the daily human diet is 400 g. In Europe, the estimated daily consumption of fructans is 3–11 g/d (34). Therefore, the dose of fructans in this study seems not to be excessive. In addition, the experiment using diets containing 5% fructan is conservative in rodent experiments. FOS, a low-DP fructan, is fermented more rapidly and produces more gas than inulin, a high-DP fructan (35). The $AUC_{0–28d}$ in the HFF group was 174% of that in the HFI group ($P = 0.051$). Low DP fructan could be a good source to supply a large amount of H_2 in the body. Also, although no significant difference was observed, adipose mRNA abundance of Il6, Ccl2, and Nfκb1 in the HFF group was 60%, 69%, 75%, and 93% of the respective amounts in the HFI group. The lower the DP of fructan, the adipose mRNA abundance might decrease due to the rapid fermentation rate of low-DP fructan.

In our previous study, we found that the ability to generate H_2 varied among the rats, even after administration of the same fermentation substrate (13). In Expt. 3, a significant difference in H_2 excretion at specific time points was not detected between the HFF and HFI groups, although the number of rats in the groups increased to $n = 8$. In Expt. 3, the minimum sample size required to define the difference between the both groups was calculated as 18 rats per group by a power analysis based on the data of H_2 excretion. A further study with a larger sample size is required to evaluate the effect of colonic H_2 generated from fructan.

This is the first study to our knowledge showing the localization of H_2 generated by fermentation in the large intestine to the adipose tissue due to diffusion into the abdominal cavity. In addition, the reduced cytokine expression by FOS and inulin might be partially dependent on increased production of H_2 in the large intestine. In the present study, intensive cytokine expression was not induced in rats fed the HFC diet, because rats were fed for only a short period of 4 wk. The effect of FOS and inulin on adipose cytokine expression might be clearly observed if dietary-induced obesity occurred through a longer feeding period. Colonic H_2 may have important implications in the suppressive effect against metabolic syndrome via oxidative stress and inflammation.

Acknowledgments

N.N. designed the research; N.N., H.T., M.A., T.Y., and M.F. conducted the research; N.N. and M.F analyzed the dataset; N.N. wrote the manuscript; and N.N. had primary responsibility for the final content. All authors were involved in designing the study, reviewing and interpreting the results, and drafting the manuscript. All authors read and approved the final manuscript.

Literature Cited

1. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest. 2004;114:1752–61.
2. Roberts CK, Sindhu KK. Oxidative stress and metabolic syndrome. Life Sci. 2009;84:705–12.
3. Mayne ST. Antioxidant nutrients and chronic disease: use of biomarkers of exposure and oxidative stress status in epidemiologic research. J Nutr. 2003;133 Suppl 3:S933–40.
4. Ohsawa I, Ishikawa M, Takahashi K, Watanabe M, Nishimaki K, Yamagata K, Katsura K, Katayama Y, Asoh S, Ohta S. Hydrogen acts as a therapeutic antioxidant by selectively reducing cytotoxic oxygen radicals. Nat Med. 2007;13:688–94.
5. Hayashida K, Sano M, Ohsawa I, Shinmura K, Tamaki K, Kimura K, Endo J, Katayama T, Kawamura A, Kohsaka S, et al. Inhalation of hydrogen gas reduces infarct size in the rat model of myocardial ischemia-reperfusion injury. Biochem Biophys Res Commun. 2008;373:30–5.
6. Kajiya M, Sato K, Silva MJ, Ouhara K, Do PM, Shanmugam KT, Kawai T. Hydrogen from intestinal bacteria is protective for Concanavalin A-induced hepatitis. Biochem Biophys Res Commun. 2009;386:316–21.
7. Nagata K, Nakashima-Kamimura N, Mikami T, Ohsawa I, Ohta S. Consumption of molecular hydrogen prevents the stress-induced impairments in hippocampus-dependent learning tasks during chronic physical restraint in mice. Neuropsychopharmacology. 2009;34:501–8.
8. Zheng X, Mao Y, Cai J, Li Y, Liu W, Sun P, Zhang JH, Sun X, Yuan H. Hydrogen-rich saline protects against intestinal ischemia/reperfusion injury in rats. Free Radic Res. 2009;43:478–84.
9. Chen H, Sun YP, Hu PF, Liu WW, Xiang HG, Li Y, Yan RL, Su N, Ruan CP, Sun XJ, et al. The effects of hydrogen-rich saline on the contractile and structural changes of intestine induced by ischemia-reperfusion in rats. J Surg Res. 2011;167:316–22.
10. Bond JH, Levitt MD. Effect of dietary fiber on intestinal gas production and small bowel transit time in man. Am J Clin Nutr. 1978;31:S169–74.
11. Cummings JH. Short chain fatty acids in the human colon. Gut. 1981;22:763–79.
12. Muir JG, Lu ZX, Young GP, Cameron-Smith D, Collier GR, O'Dea K. Resistant starch in the diet increases breath hydrogen and serum acetate in human subjects. Am J Clin Nutr. 1995;61:792–9.
13. Nishimura N, Tanabe H, Sasaki Y, Makita Y, Ohata M, Yokoyama S, Asano M, Yamamoto T, Kiriyama S. Pectin and high-amylose maize starch increase caecal hydrogen production and relieve hepatic ischaemia-reperfusion injury in rats. Br J Nutr. 2012;107:485–92.
14. Vibrans FC. The solubility of gases in edible fats and oils. Oil & Soap. 1935;12:14–5.
15. Schaffer PS, Haller HS. The solubility of gases in butter oil, cottonseed oil, and lard. Oil & Soap. 1943;20:161–2.
16. Oku T, Nakamura S. Comparison of digestibility and breath hydrogen gas excretion of fructo-oligosaccharide, galactosyl-sucrose, and isomalto-oligosaccharide in healthy human subjects. Eur J Clin Nutr. 2003;57:1150–6.
17. Rumessen JJ, Bode S, Hamberg O, Gudmand-Hoyer E. Fructans of Jerusalem artichokes: intestinal transport, absorption, fermentation, and influence on blood glucose, insulin, and C-peptide responses in healthy subjects. Am J Clin Nutr. 1990;52:675–81.
18. NCBI-Nucleotide. Rattus norvegicus strain BN/SsNHsdMCW chromosome 2, Rnor_5.0.; 2012 [cited 2012 Nov 20]. Available from: http://www.ncbi.nlm.nih.gov/nuccore/NC_005101.3.
19. Guo X, Xia X, Tang R, Zhou J, Zhao H, Wang K. Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. Lett Appl Microbiol. 2008;47:367–73.
20. Hoshi S, Sakata T, Mikuni K, Hashimoto H, Kimura S. Galactosylsucrose and xylosylfructoside alter digestive tract size and concentrations of cecal organic acids in rats fed diets containing cholesterol and cholic acid. J Nutr. 1994;124:52–60.
21. Buchholz BM, Kaczorowski DJ, Sugimoto R, Yang R, Wang Y, Billiar TR, McCurry KR, Bauer AJ, Nakao A. Hydrogen inhalation ameliorates oxidative stress in transplantation induced intestinal graft injury. Am J Transplant. 2008;8:2015–24.
22. Cai J, Kang Z, Liu WW, Luo X, Qiang S, Zhang JH, Ohta S, Sun X, Xu W, Tao H, et al. Hydrogen therapy reduces apoptosis in neonatal hypoxia-ischemia rat model. Neurosci Lett. 2008;441:167–72.
23. Chen H, Sun YP, Li Y, Liu WW, Xiang HG, Fan LY, Sun Q, Xu XY, Cai JM, Ruan CP, et al. Hydrogen-rich saline ameliorates the severity of l-arginine-induced acute pancreatitis in rats. Biochem Biophys Res Commun. 2010;393:308–13.
24. Fu Y, Ito M, Fujita Y, Ito M, Ichihara M, Masuda A, Suzuki Y, Maesawa S, Kajita Y, Hirayama M, et al. Molecular hydrogen is protective against 6-hydroxydopamine-induced nigrostriatal degeneration in a rat model of Parkinson's disease. Neurosci Lett. 2009;453:81–5.
25. Fukuda K, Asoh S, Ishikawa M, Yamamoto Y, Ohsawa I, Ohta S. Inhalation of hydrogen gas suppresses hepatic injury caused by ischemia/reperfusion through reducing oxidative stress. Biochem Biophys Res Commun. 2007;361:670–4.
26. Tanabe H, Sasaki Y, Yamamoto T, Kiriyama S, Nishimura N. Suppressive effect of high hydrogen generating high amylose cornstarch on subacute hepatic ischemia-reperfusion injury in rats. Biosci Microbiota Food Health. 2012;31:103–8.
27. Kadowaki T, Yamauchi T, Kubota N, Hara K, Ueki K, Tobe K. Adiponectin and adiponectin receptors in insulin resistance, diabetes, and the metabolic syndrome. J Clin Invest. 2006;116:1784–92.
28. Cani PD, Neyrinck AM, Fava F, Knauf C, Burcelin RG, Tuohy KM, Gibson GR, Delzenne NM. Selective increases of bifidobacteria in gut microflora improve high-fat-diet-induced diabetes in mice through a mechanism associated with endotoxaemia. Diabetologia. 2007;50:2374–83.
29. Hansen CH, Frokiaer H, Christensen AG, Bergstrom A, Licht TR, Hansen AK, Metzdorff SB. Dietary xylooligosaccharide downregulates IFN-gamma and the low-grade inflammatory cytokine IL-1beta systemically in mice. J Nutr. 2013;143:533–40.
30. Al-Lahham SH, Roelofsen H, Priebe M, Weening D, Dijkstra M, Hoek A, Rezaee F, Venema K, Vonk RJ. Regulation of adipokine production in human adipose tissue by propionic acid. Eur J Clin Invest. 2010;40:401–7.
31. Xiong Y, Miyamoto N, Shibata K, Valasek MA, Motoike T, Kedzierski RM, Yanagisawa M. Short-chain fatty acids stimulate leptin production

in adipocytes through the G protein-coupled receptor GPR41. Proc Natl Acad Sci USA. 2004;101:1045–50.

32. Pomare EW, Branch WJ, Cummings JH. Carbohydrate fermentation in the human colon and its relation to acetate concentrations in venous blood. J Clin Invest. 1985;75:1448–54.
33. van Eijk HM, Bloemen JG, Dejong CH. Application of liquid chromatography-mass spectrometry to measure short chain fatty acids in blood. J Chromatogr B Analyt Technol Biomed Life Sci. 2009;877:719–24.
34. van Loo J, Coussement P, de Leenheer L, Hoebregs H, Smits G. On the presence of inulin and oligofructose as natural ingredients in the western diet. Crit Rev Food Sci Nutr. 1995;35:525–52.
35. Hernot DC, Boileau TW, Bauer LL, Middelbos IS, Murphy MR, Swanson KS, Fahey GC Jr. In vitro fermentation profiles, gas production rates, and microbiota modulation as affected by certain fructans, galactooligosaccharides, and polydextrose. J Agric Food Chem. 2009; 57:1354–61.